



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, G01N 33/53	A1	(11) International Publication Number: WO 99/10534 (43) International Publication Date: 4 March 1999 (04.03.99)
(21) International Application Number: PCT/US98/17280 (22) International Filing Date: 20 August 1998 (20.08.98) (30) Priority Data: 60/056,734 22 August 1997 (22.08.97) US (71) Applicant: CENTER FOR BLOOD RESEARCH, INC. [US/US]; 800 Huntington Avenue, Boston, MA 02115 (US). (72) Inventors: GUTIERREZ-RAMOS, Jose-Carlos; 70A Pleasant Street, Marblehead, MA 01945 (US). PELED, Amnon; 77 Trowbridge Street, Cambridge, MA 02138 (US). QUACK-ENBUSH, Elizabeth, J.; Unit 2, 625 Tremont Street, Boston, MA 02118 (US). (74) Agent: GREER, Helen; Banner & Witcoff, Ltd., 28 State Street, Boston, MA 02109 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DIAGNOSIS AND TREATMENT OF DISEASES WITH EOTAXIN AND ANTAGONISTS AND AGONISTS THEREOF		
(57) Abstract A method for determining if a mammal is at risk for a disease associated with abnormal levels of myeloid cells is described. A mammal is provided and an aspect of eotaxin metabolism or structure is evaluated in the mammal. An abnormality in the aspect of eotaxin metabolism or structure is diagnostic of being at risk for a disease associated with abnormal levels of myeloid cells. Also described are methods for evaluating an agent for use in modulating the level of myeloid cells in a mammal, methods for treating a disease associated with high or low levels of myeloid cells in a mammal, methods for altering the proliferation or differentiation of myeloid progenitors and methods for monitoring a therapeutic treatment for a disease affecting the level of myeloid cells in a mammal. Pharmaceutical compositions are also provided.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DIAGNOSIS AND TREATMENT OF DISEASES WITH EOTAXIN AND ANTAGONISTS AND AGONISTS THEREOF

5 This application claims the benefit of U.S. Provisional Application No. 60/056,734 filed August 22, 1997.

The U.S. Government may have rights in this invention pursuant to Grant No. HL148675-02 awarded by the National Institutes of Health.

10 **Field of the Invention**

This invention relates generally to treatments, diagnoses and therapeutic agents for diseases associated with abnormal levels of myeloid cells, utilizing the cytokine eotaxin or antagonists or agonists thereof.

15 **Background of the Invention**

Many serious diseases are associated with abnormal levels of myeloid cells in the blood. Increased levels of myeloid cells accompany, e.g., inflammatory or autoimmune diseases, e.g., allergic inflammation, systemic lupus erythematosus and rheumatoid arthritis. Decreased levels of myeloid cells accompany, e.g., myeloid leukemia, mast cell deficiencies, myelodysplastic
20 syndromes and conditions resulting from myeloablative treatments, e.g., chemotherapy or radiotherapy.

During acute and chronic inflammatory processes the bone marrow produces and exports extra mature leukocytes to the inflammation site. It was previously thought that certain cytokines, such as interleukin-5, interleukin-3 and granulocyte macrophage stimulating factor,
25 played a critical role in controlling the proliferation and differentiation of hematopoietic progenitors in the bone marrow during inflammation. It has recently been reported, however, that hematopoiesis can take place normally in the complete absence of signaling events mediated by these cytokines both in steady state and during hematopoietic stress. Nishinakamura et al., Blood 88:2458-2464 (1996).

30 **Summary of the Invention**

It is an object of the invention to provide an efficacious factor for inducing proliferation and differentiation of myeloid progenitors.

It is yet another object of the invention to utilize eotaxin or fragments or analogs thereof,

or antagonists or agonists, to treat and/or diagnose diseases associated with abnormal levels of myeloid cells.

Still another object of the invention is to provide a method for evaluating an agent for use in treating diseases associated with abnormal levels of myeloid cells.

5 In one aspect, the invention features a method for determining if a mammal is at risk for a disease associated with abnormal levels of myeloid cells, e.g., granulocytes, macrophages or mast cells. A mammal is provided. An aspect of eotaxin metabolism or structure is evaluated in the mammal. An abnormality in the aspect of eotaxin metabolism or structure is diagnostic of being at risk for a disease associated with abnormal levels of myeloid cells.

10 In certain embodiments, the abnormal level of myeloid cells is an increased level. Diseases associated with an increased level of myeloid cells include, e.g., autoimmune or inflammatory diseases, e.g., allergic inflammation, systemic lupus erythematosus and rheumatoid arthritis. In certain embodiments, the abnormal level of myeloid cells is a decreased level. Examples of such diseases include certain myeloid leukemias, mast cell deficiencies,
15 myelodysplastic syndromes, e.g., that are accompanied by marrow aplasia, and conditions resulting from myeloablative treatments, e.g., chemotherapy or radiotherapy.

Another aspect of the invention is a method for evaluating an agent for use in modulating the level of myeloid cells in a mammal. A mammalian cell or mammal having a wild-type pattern of eotaxin metabolism, or a non-wild type pattern of eotaxin metabolism, e.g., resulting
20 from overexpression or underexpression of eotaxin, is provided. An agent is provided. The agent is administered to the cell or mammal in a therapeutically effective amount. The effect of the agent on an aspect of eotaxin metabolism is evaluated. A change in the aspect of eotaxin metabolism is indicative of the usefulness of the agent in modulating the level of myeloid cells in a mammal.

25 Another aspect of the invention is the agent so identified as being useful in modulating the level of myeloid cells in a mammal.

Another aspect of the invention is a method for treating a disease associated with high levels of myeloid cells in a mammal, e.g., an autoimmune or inflammatory disease. A mammal in need of treatment for a disease associated with high levels of myeloid cells is provided. An
30 agent, e.g., an antagonist or agonist, capable of altering an aspect of eotaxin metabolism or structure is provided. The agent is administered to the mammal in a therapeutically effective amount such that treatment of the disease associated with high levels of myeloid cells in the

mammal occurs.

Another aspect of the invention is a method for treating a disease associated with low levels of myeloid cells. A mammal in need of treatment for a disease associated with low levels of myeloid cells is provided. Eotaxin or a biologically active analog or fragment thereof is
5 provided. The eotaxin or biologically active analog or fragment is administered to the mammal in a therapeutically effective amount such that treatment of the disease associated with low levels of myeloid cells occurs.

Another aspect of the invention is a method for altering the proliferation or differentiation of myeloid progenitors, e.g., increasing production of macrophages or granulocytes, in a
10 mammal. A mammal having myeloid progenitors is provided. The mammal is in need of altering the proliferation or differentiation of the myeloid progenitors. Eotaxin, a biologically active fragment thereof, a biologically active analog thereof, an antagonist or an agonist, is provided. This compound is administered to the mammal under conditions which allow the compound to alter the proliferation or differentiation of the myeloid progenitors.

15 Another aspect of the invention is a method for monitoring a therapeutic treatment for a disease affecting the level of myeloid cells in a mammal, the disease being associated with abnormal levels of an aspect of eotaxin metabolism in the mammal. The levels of an aspect of eotaxin metabolism in a plurality of biological samples obtained at different time points from a mammal undergoing a therapeutic treatment for the disease affecting the level of myeloid cells is
20 evaluated.

Another aspect of the invention is a pharmaceutical composition for treating a disease associated with low levels of myeloid cells in a mammal, comprising a therapeutically effective amount of eotaxin or a biologically active fragment or analog thereof, the eotaxin or biologically active fragment or analog thereof being capable of stimulating the proliferation or differentiation
25 of myeloid progenitors so as to result in treatment of the disease associated with low levels of myeloid cells in the mammal, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a pharmaceutical composition for treating an autoimmune or inflammatory disease in a mammal comprising a therapeutically effective amount of an agent, the agent being capable of altering an aspect of eotaxin metabolism or structure in
30 the mammal so as to result in treatment of the autoimmune or inflammatory disease in the mammal, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a method of making an altered eotaxin polypeptide

having an antagonist or agonist activity so as to alter the proliferation or differentiation of myeloid progenitors in a mammal. An eotaxin polypeptide is provided. The amino acid sequence of the eotaxin polypeptide is altered. The altered eotaxin polypeptide is tested for an effect on the proliferation or differentiation of myeloid progenitors. A change in the proliferation or differentiation is indicative of an eotaxin polypeptide having an antagonist or agonist activity.

Another aspect of the invention is a method for evaluating an agent for the ability to alter the binding of eotaxin polypeptide to a binding molecule. An agent is provided. An eotaxin polypeptide is provided. A binding molecule is provided. The agent, eotaxin polypeptide and binding molecule are combined. The formation of a complex comprising the eotaxin polypeptide and binding molecule is detected. An alteration in the formation of the complex in the presence of the agent as compared to in the absence of the agent is indicative of the agent altering the binding of the eotaxin polypeptide to the binding molecule.

Another aspect of the invention is the agent so identified as being able to alter the binding of eotaxin polypeptide to a binding molecule.

Another aspect of the invention also includes a method for evaluating an agent for the ability to bind to eotaxin polypeptide. An agent is provided. Eotaxin polypeptide is provided. The agent is contacted with the eotaxin polypeptide. The ability of the agent to bind to the eotaxin polypeptide is evaluated.

Another aspect of the invention is the agent so identified as being able to bind to eotaxin polypeptide.

Another aspect of the invention is a method for evaluating an agent for the ability to bind to a nucleic acid encoding an eotaxin regulatory sequence. An agent is provided. A nucleic acid encoding an eotaxin regulatory sequence is provided. The agent is contacted with the nucleic acid. The ability of the agent to bind to the nucleic acid is evaluated.

Yet another aspect of the invention is the agent so identified as being able to bind to a nucleic acid encoding an eotaxin regulatory sequence.

The above and other features, objects and advantages of the present invention will be better understood by a reading of the following specification.

30

Detailed Description

This invention provides a method for determining if a mammal is at risk for a disease associated with abnormal levels of myeloid cells. A mammal is provided. An aspect of eotaxin

metabolism or structure is evaluated in the mammal. An abnormality in the aspect of eotaxin metabolism or structure is diagnostic of being at risk for a disease associated with abnormal levels of myeloid cells.

By myeloid cells are meant blood cells with immune function that do not have a clonotypic receptor, e.g., T cell receptor or immunoglobulin/B cell receptor, and are not derived from a lymphoid progenitor. Examples of myeloid cells include macrophages, granulocytes and mast cells.

A mast cell is a specific type of myeloid cell that is particularly important in allergic reactions. Mast cells are bone-marrow derived cells that participate in a wide array of immune responses to pathogens, e.g., contact allergens, aerosolized allergens, parasites and tumors. Mast cells can be present in an embryo or adult. They are normally found in the connective and mucosal tissues, e.g., skin, nasal polyps and gut. They are generally characterized by secretory granules that are metachromatic when stained with cationic dyes, and by their granular contents. Their granules contain histamine and enzymes called serine proteases, both of which can be released at sites of allergic responses and inflammation to build a defense against foreign antigens. Mast cells in the lung figure prominently in asthmatic responses, while mast cells in the gut can react against invading intestinal parasites. The variety of chemical mediators that is stored in the granules of mast cells can be altered by the environment to which the mast cells are exposed. Eotaxin is capable of altering protease expression patterns in mast cells. For example, the response of mast cells to eotaxin can be altered at sites of inflammation that involve high levels of eotaxin, such as asthma.

By mammal is meant human as well as non-human mammals. Non-human mammals include, e.g., rodents, e.g., mice or rats, rabbits, monkeys and pigs. A mammal also includes transgenic non-human mammals. The term transgenic mammal is meant to include a mammal which has gained new genetic information from the introduction of foreign DNA, i.e., partly or entirely heterologous DNA, into the DNA of its cells; or introduction of a lesion, e.g., an in vitro induced mutation, e.g., a deletion or other chromosomal rearrangement into the DNA of its cells; or introduction of homologous DNA into the DNA of its cells in such a way as to alter the genome of the cell into which the DNA is inserted, e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout. The mammal may include a transgene in all of its cells including germ line cells, or in only one or some of its cells.

Transgenic mammals of the invention can serve as a model for studying diseases associated with

abnormal levels of myeloid cells.

In certain embodiments, the determination for being at risk for a disease associated with abnormal levels of myeloid cells is done in a prenatal mammal.

In certain embodiments, the abnormal level of myeloid cells is an increased level.

- 5 Diseases associated with an increased level of myeloid cells include, e.g., autoimmune or inflammatory diseases. Examples of such diseases include allergic inflammation, e.g., lung allergic inflammation, systemic lupus erythematosus and rheumatoid arthritis.

In certain embodiments, the abnormal level of myeloid cells is a decreased level.

- 10 Examples of such diseases include myeloid leukemia, mast cell deficiencies, myelodysplastic syndromes, e.g., that are accompanied by marrow aplasia, and conditions resulting from myeloablative treatments, e.g., chemotherapy or radiotherapy.

- In certain embodiments, the disease involving abnormal levels of myeloid cells is due to abnormal levels of mast cells. Mast cells accumulate and are activated in, e.g., allergic asthma, rhinitis, urticaria and eczema (atopic dermatitis). Other allergic reactions, including, e.g., hay
15 fever, angioneurotic edema and anaphylactic states, are also associated with the degranulation of mast cells. Parasitic infections with mast cell involvement include, e.g., trichinella and strongyloides, microfilariasis. Skin diseases involving mast cells include, e.g., dermatitis and pemphigus. Systemic mastocytosis, an abnormal overabundance of mast cells in the bone marrow and tissues is found in leukemic and dysmyelopoietic states. Pronounced mastocytosis
20 occurs in mammary carcinoma, neurofibromas, fibrous histiocytoma and in some carcinomas. There are also rare benign and malignant mast cell neoplasms: mast cell reticulosis, mast cell sarcoma and mast cell leukemia. Hypereosinophilia syndromes, e.g., periarteritis nodosa, eosinophilic granuloma, and chronic eosinophilic pneumonia also involve eotaxin and mast cells.

- Eotaxin is a CC chemokine that has been shown to be an eosinophil-specific
25 chemoattractant that is highly expressed during acute and chronic allergic processes. The mouse and human eotaxin gene have been cloned (Gonzalo et al., Immunity 4:1-20 (1996); Ponath et al., J. Clin. Invest. 97:1-9 (1996)). This invention demonstrates that eotaxin can also act as a proliferation and differentiation factor for myeloid progenitors, resulting in the production of myeloid cells, e.g., macrophages, granulocytes and/or mast cells.

- 30 By myeloid progenitors is meant a cell that has the potential of generating myeloid cells. Myeloid progenitors can be present in an embryo or an adult. Myeloid progenitors include, e.g., mast cell progenitors.

By eotaxin metabolism is meant any aspect of the production, differentiation, release, expression, function, action, interaction or regulation of eotaxin, or any change that eotaxin induces in the production, differentiation, release, expression, function, action, interaction or regulation of other substances. The metabolism of eotaxin includes modifications, e.g., covalent or non-covalent modifications, of eotaxin polypeptide, and covalent or non-covalent modifications that eotaxin induces in other substances. The metabolism of eotaxin also includes changes in the distribution, concentration, activation or phosphorylation of eotaxin polypeptide, and changes that eotaxin induces in the distribution, concentration, activation, or phosphorylation of other substances. The terms polypeptides, peptides and proteins are used interchangeably herein.

Any aspect of eotaxin metabolism can be evaluated. The methods used are standard techniques known to those skilled in the art and can be found in standard references, e.g., Ausubel et al., ed., Current Protocols in Mol. Biology, New York: John Wiley & Sons, 1990. (Drewes et al., Mol. and Cell. Biol. 16(3):925-931 (1996)). Preferred examples of eotaxin metabolism that can be evaluated include the binding activity of eotaxin polypeptide to a binding molecule; the transactivation activity of eotaxin polypeptide on a target gene; the level of eotaxin protein; or the level of eotaxin mRNA. By binding molecule is meant any molecule to which eotaxin can bind, e.g., a nucleic acid, e.g., a DNA regulatory region, a protein, a metabolite, a peptide mimetic, a non-peptide mimetic, an antibody, or any other type of ligand. The binding molecule can be natural or artificial. In certain embodiments, the binding molecule is a receptor of eotaxin. An example of an eotaxin receptor includes chemokine receptor CCR-3. (Tenschler et al., Blood 88:3195 (1996); Rothenberg et al., Mol. Med. 2:334 (1996); Ponath et al., J. Exp. Med. 183:2437 (1996); Daugherty et al., J. Exp. Med. 183:2349 (1996)). Binding can be shown, e.g., by electrophoretic mobility shift analysis (EMSA). (Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). Transactivation of a target gene by eotaxin can be determined, e.g., in a transient transfection assay in which the promoter of the target gene is linked to a reporter gene, e.g., β -galactosidase or luciferase, and co-transfected with an eotaxin expression vector. (Gonzalo et al., J. Clin. Invest. 98:1 (1996); Gonzalo et al., Immunity 4:1 (1996)). Levels of eotaxin protein or mRNA can, e.g., be measured in a sample, e.g., in circulating blood cells. (Gonzalo et al., J. Clin. Invest. 98:1 (1996); Gonzalo et al., Immunity 4:1 (1996)).

In certain embodiments, an aspect of eotaxin structure is evaluated, e.g., eotaxin gene

structure or eotaxin protein structure. For example, primary, secondary or tertiary structures can be evaluated. For example, the DNA sequence of the gene is determined and/or the amino acid sequence of the protein is determined. (Gonzalo et al., J. Clin. Invest. 98:1 (1996); Gonzalo et al., Immunity 4:1 (1996)). Standard cloning and sequencing methods can be used as are known to those skilled in the art. In certain embodiments, the binding activity of an antisense nucleic acid with the cellular eotaxin mRNA and/or genomic DNA is determined using standard methods known to those skilled in the art so as to detect the presence or absence of the target mRNA or DNA sequences to which the antisense nucleic acid would normally specifically bind. (Gonzalo et al., J. Clin. Invest. 98:1 (1996); Gonzalo et al., Immunity 4:1 (1996)).

In preferred embodiments, the abnormality in the aspect of metabolism or structure is a mutation in a gene encoding eotaxin or a receptor of eotaxin. In other preferred embodiments, the abnormality in the aspect of metabolism or structure is abnormal polypeptide or RNA levels of eotaxin or of a receptor of eotaxin.

The invention also includes a method for evaluating an agent for use in modulating the level of myeloid cells in a mammal. A mammalian cell or mammal is provided. An agent is provided. The agent is administered to the cell or mammal in a therapeutically effective amount. The effect of the agent on an aspect of eotaxin metabolism is evaluated. A change in the aspect of eotaxin metabolism is indicative of the usefulness of the agent in modulating the level of myeloid cells in a mammal.

By cell is meant a cell or a group of cells, or a cell that is part of a mammal. The cell can be a human or non-human cell. Cell is also meant to include a transgenic cell. The cell can be obtained, e.g., from a culture or from a mammal. Mammals are meant to include, e.g., natural mammals and non-human transgenic mammals. In certain embodiments, the transgenic cell or non-human transgenic mammal has an eotaxin transgene, or fragment or analog thereof. In certain embodiments, the transgenic cell or non-human transgenic mammal has a mutation in the eotaxin gene. In certain embodiments, the transgenic cell or non-human transgenic mammal has a knockout for the eotaxin gene.

In certain embodiments, the myeloid cells are mast cells, granulocytes, macrophages or combinations thereof.

In certain embodiments, the mammalian cell or mammal that is provided has a wild type pattern of eotaxin metabolism. In other embodiments, the mammalian cell or mammal that is provided has a non-wild type pattern of eotaxin metabolism, e.g., which results from under-

expression, over-expression, no expression, or a temporal, site or distribution change. Such a non-wild type pattern can result, e.g., from one or more mutations in the eotaxin gene, in a binding molecule gene, or in any other gene which directly or indirectly affects eotaxin metabolism. A mutation is meant to include, e.g., an alteration, e.g., in gross or fine structure, in a nucleic acid. Examples include single base pair alterations, e.g., missense or nonsense mutations, frameshifts, deletions, insertions, duplications, inversions and translocations. Mutations can be dominant or recessive. Mutations can be homozygous or heterozygous. In a preferred embodiment, the aspect of eotaxin metabolism that is evaluated is a parameter related to the level of myeloid cells.

10 In certain embodiments, the method employs two phases for evaluating an agent for use in modulating the level of myeloid cells, an initial in vitro phase and then an in vivo phase. The agent is administered to the mammalian cell in vitro, and if a change in an aspect of eotaxin metabolism occurs, then the agent is further administered to a mammal in a therapeutically effective amount and evaluated in vivo for an effect of the agent on an aspect of eotaxin
15 metabolism. A change in the aspect of eotaxin metabolism is indicative of the usefulness of the agent in modulating the level of myeloid cells in a mammal.

In certain embodiments, the method further comprises providing stem cell factor (SCF) or a biologically active fragment or analog thereof, and administering the SCF or biologically active fragment or analog thereof in a therapeutically effective amount. Preferably, the SCF and
20 agent are administered together. The SCF and agent can have an additive or synergistic effect in modulating the level of myeloid cells.

An agent is meant to include, e.g., any substance, e.g., a drug. The agent of this invention preferably can change an aspect of eotaxin metabolism. Such change can be the result of any of a variety of events, including, e.g., preventing, reducing or increasing interaction between
25 eotaxin and a binding molecule; inactivating or activating eotaxin and/or the binding molecule, e.g., by cleavage or other modification; altering the affinity of eotaxin and the binding molecule for each other; diluting out eotaxin and/or the binding molecule; preventing or promoting expression of eotaxin and/or the binding molecule; reducing or increasing synthesis of eotaxin and/or the binding molecule; synthesizing an abnormal eotaxin and/or binding molecule;
30 synthesizing an alternatively spliced eotaxin and/or binding molecule; preventing or reducing proper conformational folding of eotaxin and/or the binding molecule; modulating the binding properties of eotaxin and/or the binding molecule; interfering with signals that are required to

activate or deactivate eotaxin and/or the binding molecule; activating or deactivating eotaxin and/or the binding molecule at the wrong time; or interfering with other receptors, ligands or other molecules which are required for the normal synthesis or functioning of eotaxin and/or the binding molecule. In certain preferred embodiments, the binding molecule is a receptor for eotaxin.

Examples of agents include eotaxin polypeptide or a biologically active fragment or analog thereof; a nucleic acid encoding eotaxin polypeptide or a regulatory sequence, or a biologically active fragment thereof; a binding molecule for eotaxin polypeptide, e.g., an eotaxin receptor; a binding molecule for eotaxin nucleic acid, the eotaxin nucleic acid being, e.g., a nucleic acid comprising a regulatory region for eotaxin or a nucleic acid comprising a structural region for eotaxin or a biologically active fragment of eotaxin; an antisense nucleic acid; a mimetic of eotaxin or a binding molecule; an antibody for eotaxin or a binding molecule; a metabolite; or an inhibitory carbohydrate or glycoprotein. In certain embodiments, the agent is a natural ligand for eotaxin. In certain embodiments, the agent is an artificial ligand for eotaxin.

The agent can be, e.g., an antagonist, agonist or super agonist.

Eotaxin polypeptide can be obtained, e.g., from purification or secretion of naturally occurring eotaxin, e.g., from bronchoalveolar lavage fluid of asthmatic mammals, from recombinant eotaxin, or from synthesized eotaxin, by methods known to those skilled in the art. (See, e.g., Gonzalo et al., J. Clin. Invest. 98:1 (1996); Gonzalo et al., Immunity 4:1 (1996)).

By analog is meant a compound that differs from naturally occurring eotaxin in amino acid sequence or in ways that do not involve sequence, or both. Analogs of the invention generally exhibit at least about 90% homology, preferably at least about 95% homology, and most preferably at least about 99% homology, with a segment of about 20 amino acid residues, preferably with more than about 40 amino acid residues, and more preferably yet with substantially the entire sequence of a naturally occurring eotaxin sequence. Non-sequence modifications include, e.g., in vivo or in vitro chemical derivatizations of eotaxin. Non-sequence modifications include, e.g., changes in phosphorylation, acetylation, methylation, carboxylation, or glycosylation. Methods for making such modifications are known to those skilled in the art. For example, phosphorylation can be modified by exposing eotaxin to phosphorylation-altering enzymes, e.g., kinases or phosphatases.

Preferred analogs include eotaxin or biologically active fragments thereof, whose sequences differ from the wild-type sequence by one or more conservative amino acid

substitutions or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish eotaxin biological activity. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other examples of conservative substitutions are shown in Table 1.

Table 1

CONSERVATIVE AMINO ACID SUBSTITUTIONS

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	G	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β -Ala Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Other analogs within the invention include, e.g., those with modifications which increase peptide stability. Such analogs may contain, e.g., one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are, e.g.: analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids; and cyclic analogs.

Amino acid sequence variants of a protein can be prepared by any of a variety of methods known to those skilled in the art. For example, random mutagenesis of DNA which encodes a protein or a particular domain or region of a protein can be used, e.g., PCR mutagenesis (using, e.g., reduced *Taq* polymerase fidelity to introduce random mutations into a cloned fragment of DNA; Leung et al., Technique 1:11-15 (1989)), or saturation mutagenesis (by, e.g., chemical treatment or irradiation of single-stranded DNA in vitro, and synthesis of a complementary DNA strand; Mayers et al., Science 229:242 (1985)). Random mutagenesis can also be accomplished by, e.g., degenerate oligonucleotide generation (using, e.g., an automatic DNA synthesizer to chemically synthesize degenerate sequences; Narang, Tetrahedron 39:3 (1983); Itakura et al., Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. A.G. Walton, Amsterdam: Elsevier, pp. 273-289 (1981)). Non-random or directed mutagenesis can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (i) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (ii) deleting the target residue, (iii) inserting residues of the same or a different class adjacent to the located site, or (iv) combinations of the above.

Methods for identifying desirable mutations include, e.g., alanine scanning mutagenesis (Cunningham and Wells, Science 244:1081-1085 (1989)), oligonucleotide-mediated mutagenesis (Adelman et al., DNA 2:183 (1983)); cassette mutagenesis (Wells et al., Gene 34:315 (1985)), combinatorial mutagenesis, and phage display libraries (Ladner et al., WO88/06630).

By fragment is meant some portion of the naturally occurring eotaxin polypeptide. Preferably, the fragment is at least about 20 amino acid residues, more preferably at least about 40 amino acid residues, and most preferably at least about 60 amino acid residues in length. Fragments include, e.g., truncated secreted forms, proteolytic fragments, splicing fragments, other fragments, and chimeric constructs between at least a portion of the relevant gene, e.g., eotaxin, and another molecule. Fragments of eotaxin can be generated by methods known to

those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of eotaxin can be assessed by methods known to those skilled in the art, e.g., Met-Rantes (Gonzalo et al., J. Clin. Invest. 98:1 (1996)). Also included are eotaxin fragments containing residues that are not required for biological activity of the fragment or that result from alternative mRNA

5 splicing or alternative protein processing events.

Fragments of a protein can be produced in several ways, e.g., recombinantly, by chemical synthesis, or by proteolytic digestion. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of
10 the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNAs which encode an array of fragments. DNAs which encode fragments of a protein can also be generated, e.g., by random shearing, restriction digestion or a combination of the above-described methods. For example, fragments of eotaxin can be made by expressing eotaxin DNA which has been manipulated in vitro to encode the
15 desired fragment. Fragments can also be chemically synthesized using techniques known in the art, e.g., conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, peptides of the present invention can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

Eotaxin or a biologically active fragment or analog thereof, or a binding molecule or a
20 biologically active fragment or analog thereof, can, e.g., compete with its cognate molecule for the binding site on the complementary molecule, and thereby reduce or eliminate binding between eotaxin and the cellular binding molecule.

An agent can also be a nucleic acid used as an antisense molecule. Antisense therapy is meant to include, e.g., administration or in situ generation of oligonucleotides or their derivatives
25 which specifically hybridize, e.g., bind, under cellular conditions, with the cellular mRNA and/or genomic DNA encoding eotaxin polypeptide, or a mutant thereof, so as to inhibit expression of the encoded protein, e.g., by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In certain
30 embodiments, the antisense construct binds to a naturally-occurring sequence of an eotaxin gene which, e.g., is involved in expression of the gene. These sequences include, e.g., start codons, stop codons, and RNA primer binding sites. In other embodiments, the antisense construct binds

to a nucleotide sequence which is not present in the wild type gene. For example, the antisense construct can bind to a region of an eotaxin gene which contains an insertion of an exogenous, non-wild type sequence. Alternatively, the antisense construct can bind to a region of an eotaxin gene which has undergone a deletion, thereby bringing two regions of the gene together which are not normally positioned together and which, together, create a non-wild type sequence. When administered in vivo to a subject, antisense constructs which bind to non-wild type sequences provide the advantage of inhibiting the expression of a mutant eotaxin gene, without inhibiting expression of any wild type eotaxin gene.

An antisense construct of the present invention can be delivered, e.g., as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes eotaxin polypeptide. An alternative is that the antisense construct is an oligonucleotide probe which is generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of an eotaxin gene. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA. (See, e.g., U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed. (See, e.g., Van der Krol et al., *Biotechniques* 6:958-976, (1988); Stein et al., *Cancer Res.* 48:2659-2668 (1988)).

By mimetic is meant a molecule which resembles in shape and/or charge distribution eotaxin or a binding molecule. The mimetic can be a peptide or a non-peptide. Mimetics can act as therapeutic agents because they can, e.g., competitively inhibit binding of eotaxin to a binding molecule. By employing, e.g., scanning mutagenesis, e.g., alanine scanning mutagenesis, linker scanning mutagenesis or saturation mutagenesis, to map the amino acid residues of a particular eotaxin polypeptide involved in binding a binding molecule, peptide mimetics, e.g., diazopine or isoquinoline derivatives, can be generated which mimic those residues in binding to a binding molecule, and which therefore can inhibit binding of the eotaxin to a binding molecule and thereby interfere with the function of eotaxin. For example, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (see, e.g., Freidinger et al., in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988));

azepine (see, e.g., Huffman et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988)); substituted gamma lactam rings (see, e.g., Garvey et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988)); keto-methylene pseudopeptides (see, e.g., Ewenson et al., J. Med. Chem. 29:295 (1986); Ewenson et al., in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL (1985)); β -turn dipeptide cores (see, e.g., Nagai et al., Tetrahedron Lett. 26:647 (1985); Sato et al., J. Chem. Soc. Perkin Trans. 1:1231 (1986)); or β -amino alcohols (see, e.g., Gordon et al., Biochem. Biophys. Res. Commun. 126:419 (1985); Dann et al., Biochem. Biophys. Res. Commun. 134:71 (1986)).

Antibodies are meant to include antibodies against any moiety that directly or indirectly affects eotaxin metabolism. The antibodies can be directed against, e.g., eotaxin or a binding molecule, or a subunit or fragment thereof. For example, antibodies include anti-eotaxin antibodies; anti-binding molecule antibodies; and Fab₂' fragments of the inhibitory antibody generated through, e.g., enzymatic cleavage. Both polyclonal and monoclonal antibodies can be used in this invention. Preferably, monoclonal antibodies are used. Most preferably, the antibodies have a constant region derived from a human antibody and a variable region derived from an inhibitory mouse monoclonal antibody. Antibodies to eotaxin are described in Gonzalo et al., J. Clin. Invest. 98:1 (1996).

Agents also include inhibitors of a molecule that are required for synthesis, post-translational modification, or functioning of eotaxin and/or a binding molecule, or activators of a molecule that inhibit the synthesis or functioning of eotaxin and/or the binding molecule. Agents include, e.g., cytokines, growth factors, hormones, signaling components, kinases, phosphatases, homeobox proteins, transcription factors, translation factors and post-translation factors or enzymes. Agents are also meant to include ionizing radiation, non-ionizing radiation, ultrasound and toxic agents which can, e.g., at least partially inactivate or destroy eotaxin and/or the binding molecule.

An agent is also meant to include agents which are not entirely eotaxin specific. For example, an agent may also alter other genes, proteins or factors related to hematopoietic progenitors in general, or to myeloid progenitors specifically. Such overlapping specificity may provide additional therapeutic advantage. For example, an agent may alter other chemokine interactions, e.g., stem cell factor (SCF) interactions. For example, eotaxin has been shown to act synergistically with SCF to stimulate production of granulocytes, macrophages and mast

cells. See Examples.

The invention also includes the agent so identified as being useful in modulating the level of myeloid cells in a mammal.

The invention also includes a method for treating a disease associated with high levels of myeloid cells in a mammal. A mammal in need of treatment for a disease associated with high levels of myeloid cells is provided. An agent capable of altering an aspect of eotaxin metabolism or structure is provided. The agent is administered to the mammal in a therapeutically effective amount such that treatment of the disease associated with high levels of myeloid cells in the mammal occurs.

Diseases associated with high levels of myeloid cells include, e.g., autoimmune or inflammatory diseases. Examples include allergic inflammation, e.g., lung allergic inflammation, systemic lupus, erythematosus and rheumatoid arthritis.

In certain embodiments, the myeloid cells are mast cells, granulocytes, macrophages or combinations thereof.

Treating is meant to include, e.g., preventing, treating, reducing the symptoms of, or curing the disease associated with high levels of myeloid cells. Administration of the agent can be accomplished by any method which allows the agent to reach the target cells. These methods include, e.g., injection, deposition, implantation, suppositories, oral ingestion, inhalation, topical administration, or any other method of administration where access to the target cells by the agent is obtained. Injections can be, e.g., intravenous, intradermal, subcutaneous, intramuscular or intraperitoneal. Implantation includes inserting implantable drug delivery systems, e.g., microspheres, hydrogels, polymeric reservoirs, cholesterol matrices, polymeric systems, e.g., matrix erosion and/or diffusion systems and non-polymeric systems, e.g., compressed, fused or partially fused pellets. Suppositories include glycerin suppositories. Oral ingestion doses can be enterically coated. Inhalation includes administering the agent with an aerosol in an inhalator, either alone or attached to a carrier that can be absorbed.

Administration of the agent can be alone or in combination with other therapeutic agents. In certain embodiments, the agent can be combined with a suitable carrier, incorporated into a liposome, or incorporated into a polymer release system.

In certain embodiments of the invention, the administration can be designed so as to result in sequential exposures to the agent over some time period, e.g., hours, days, weeks, months or years. This can be accomplished by repeated administrations of the agent by one of

the methods described above, or alternatively, by a controlled release delivery system in which the agent is delivered to the mammal over a prolonged period without repeated administrations. By a controlled release delivery system is meant that total release of the agent does not occur immediately upon administration, but rather is delayed for some time period. Release can occur in bursts or it can occur gradually and continuously. Administration of such a system can be, e.g., by long acting oral dosage forms, bolus injections, transdermal patches or sub-cutaneous implants.

Examples of systems in which release occurs in bursts include, e.g., systems in which the agent is entrapped in liposomes which are encapsulated in a polymer matrix, the liposomes being sensitive to a specific stimuli, e.g., temperature, pH, light or a degrading enzyme, and systems in which the agent is encapsulated by an ionically-coated microcapsule with a microcapsule core-degrading enzyme. Examples of systems in which release of the agent is gradual and continuous include, e.g., erosional systems in which the agent is contained in a form within a matrix, and diffusional systems in which the agent permeates at a controlled rate, e.g., through a polymer. Such sustained release systems can be, e.g., in the form of pellets or capsules.

The agent can be suspended in a liquid, e.g., in dissolved form or colloidal form. The liquid can be a solvent, partial solvent or non-solvent. In many cases water or an organic liquid can be used.

The agent can be administered prior to or subsequent to the appearance of disease symptoms. In certain embodiments, the agent is administered to patients with familial histories of the disease, or who have phenotypes that may indicate a predisposition to the disease, or who have been diagnosed as having a genotype which predisposes the patient to the disease.

The agent is administered to the mammal in a therapeutically effective amount. By therapeutically effective amount is meant that amount which is capable of at least partially preventing or reversing the disease associated with high levels of myeloid cells. A therapeutically effective amount can be determined on an individual basis and will be based, at least in part, on consideration of the species of mammal, the mammal's size, the mammal's age, the agent used, the type of delivery system used, the time of administration relative to the onset of disease symptoms, and whether a single, multiple, or controlled release dose regimen is employed. A therapeutically effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

Preferably, the concentration of the agent is at a dose of about 0.1 to about 1000 mg/kg

body weight, more preferably at about 0.1 to about 500 mg/kg, more preferably yet at about 0.1 to about 100 mg/kg, and most preferably at about 0.1 to about 5 mg/kg. Preferably, the agent is administered about one to about five times per day, most preferably one time per day. The specific concentration partially depends upon the particular agent used, as some are more effective than others. The dosage concentration of the agent that is actually administered is dependent at least in part upon the final concentration that is desired at the site of action, the method of administration, the efficacy of the particular agent, the longevity of the particular agent, and the timing of administration relative to the onset of the disease symptoms. Preferably, the dosage form is such that it does not substantially deleteriously affect the animal. The dosage can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

In certain embodiments, various gene constructs can be used as part of a gene therapy protocol to deliver nucleic acids encoding, e.g., either an agonistic or antagonistic form of eotaxin polypeptide or an eotaxin binding polypeptide, e.g., an eotaxin receptor. Expression vectors can be used for in vivo transfection and expression of the polypeptide in particular cell types so as to reconstitute the function of, or alternatively, abrogate the function of the polypeptide in a cell in which non-wild type eotaxin is expressed. Expression constructs of the polypeptide, and mutants thereof, may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the gene to cells in vivo. Approaches include, e.g., insertion of the subject gene in viral vectors including, e.g., recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO_4 precipitation carried out in vivo. The above-described methods are known to those skilled in the art and can be performed without undue experimentation. Since transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g., locally or systemically. Administration can be directed to one or more cell types, and to one or more cells within a cell type, so as to be therapeutically effective, by methods that are known to those skilled in the art. For example, a genetically engineered

eotaxin gene is administered to stem cells. In certain embodiments, administration is done in a prenatal mammal or embryonic cell. It will be recognized that the particular gene construct provided for in in vivo transduction of eotaxin expression is also useful for in vitro transduction of cells, such as for use in the diagnostic method described above.

5 In certain embodiments, the agent is not entirely eotaxin specific, but is also capable of altering an aspect of metabolism or structure of another gene, protein, or factor, e.g., another chemokine, e.g., stem cell factor (SCF).

The invention also includes a method for treating a disease associated with low levels of myeloid cells. A mammal in need of treatment for a disease associated with low levels of
10 myeloid cells is provided. Eotaxin or a biologically active analog or fragment thereof is provided. The eotaxin or biologically active analog or fragment is administered to the mammal in a therapeutically effective amount such that treatment of the disease associated with low levels of myeloid cells occurs. In certain embodiments, the myeloid cells are mast cells, granulocytes, macrophages or combinations thereof. Diseases associated with low levels of myeloid cells
15 include, e.g., myeloid leukemia, mast cell deficiencies, myelodysplastic syndromes, e.g., that are accompanied by marrow aplasia, and conditions resulting from myeloablative treatments, e.g., chemotherapy or radiotherapy.

In certain embodiments, the method further comprises providing SCF or a biologically active fragment or analog thereof, and administering the SCF or biologically active fragment or
20 analog thereof to the mammal in a therapeutically effective amount. Preferably, the SCF or eotaxin, or biologically active analogs or fragments thereof, are administered together. The SCF and eotaxin, or biologically active analogs or fragments thereof, can have an additive or synergistic effect.

The invention also includes a method for altering the proliferation or differentiation of
25 myeloid progenitors in a mammal. A mammal having myeloid progenitors is provided. The mammal is in need of altering the proliferation or differentiation of the myeloid progenitors. Eotaxin, a biologically active fragment thereof, a biologically active analog thereof, an antagonist or an agonist, is provided. This compound is administered to the mammal under conditions which allow the compound to alter the proliferation or differentiation of the myeloid
30 progenitors. In certain embodiments, the altering of the proliferation or differentiation of the myeloid progenitors comprises increasing or decreasing production of macrophages or granulocytes. In certain embodiments, the myeloid progenitors are mast cell progenitors.

In certain embodiments, the method further comprises providing SCF or a biologically active fragment or analog thereof, and administering the SCF or biologically active fragment or analog thereof to the mammal. Preferably, the SCF or biologically active fragment or analog thereof, and the compound are administered together. The SCF and agent can have an additive or synergistic effect in altering the proliferation or differentiation of myeloid progenitors.

The invention also includes a method for monitoring a therapeutic treatment for a disease affecting the level of myeloid cells in a mammal, the disease being associated with abnormal levels of an aspect of eotaxin metabolism in the mammal. The levels of an aspect of eotaxin metabolism in a plurality of biological samples obtained at different time points from a mammal undergoing a therapeutic treatment for a disease affecting the level of myeloid cells is evaluated. The disease is associated with abnormal levels of an aspect of eotaxin metabolism in the mammal. The level of an aspect of eotaxin metabolism can be measured by any method known to those skilled in the art, including, e.g., the methods described herein. In a preferred embodiment, the aspect that is evaluated is the level of eotaxin polypeptide. In certain embodiments, the myeloid cells are mast cells, granulocytes, macrophages or combinations thereof.

The invention also includes a pharmaceutical composition for treating a disease associated with low levels of myeloid cells in a mammal, comprising a therapeutically effective amount of eotaxin or a biologically active fragment or analog thereof, the eotaxin or biologically active fragment or analog thereof being capable of stimulating the proliferation or differentiation of myeloid progenitors so as to result in treatment of the disease associated with low levels of myeloid cells in the mammal, and a pharmaceutically acceptable carrier. In certain embodiments, the myeloid cells are mast cells.

In certain embodiments, the pharmaceutical composition further comprises SCF or a biologically active fragment or analog thereof. The SCF or biologically active fragment or analog thereof is capable of increasing the stimulation of the proliferation or differentiation of the myeloid progenitors by the eotaxin or biologically active fragment or analog thereof.

The invention also includes a pharmaceutical composition for treating an autoimmune or inflammatory disease in a mammal comprising a therapeutically effective amount of an agent, the agent being capable of altering an aspect of eotaxin metabolism or structure in the mammal so as to result in treatment of the autoimmune or inflammatory disease in the mammal, and a pharmaceutically acceptable carrier. In certain embodiments, the agent is an antagonist of

eotaxin. Examples of an antagonist include an antibody for eotaxin, a fragment of eotaxin, an analog of eotaxin, a small molecule antagonist of eotaxin, a mimetic of eotaxin, an antisense molecule for eotaxin and a binding molecule for eotaxin. In certain embodiments the agent is a receptor for eotaxin. And, in certain embodiments, the agent is not entirely eotaxin specific, but is also capable of altering an aspect of metabolism or structure of another gene, protein, or factor, e.g., another chemokine, e.g., SCF.

The invention also includes a method of making an altered eotaxin polypeptide having an antagonist or agonist activity so as to alter the proliferation or differentiation of myeloid progenitors in a mammal. An eotaxin polypeptide is provided. The amino acid sequence of the eotaxin polypeptide is altered. The altered eotaxin polypeptide is tested for an effect on the proliferation or differentiation of myeloid progenitors. A change in the proliferation or differentiation is indicative of an eotaxin polypeptide having an antagonist or agonist activity. The altered eotaxin polypeptide can be generated and tested for the presence of antagonist or agonist activity by methods known to those skilled in the art without undue experimentation. (See, e.g., Gonzalo et al., J. Clin. Invest. 98:1 (1996); Gonzalo et al., Immunity 4:1 (1996)). The altered eotaxin can be made in vitro or in vivo. In certain embodiments, the myeloid progenitors are mast cell progenitors.

The invention also includes a method for evaluating an agent for the ability to alter the binding of eotaxin polypeptide to a binding molecule. An agent is provided. An eotaxin polypeptide is provided. A binding molecule is provided. The agent, eotaxin polypeptide and binding molecule are combined. The formation of a complex comprising the eotaxin polypeptide and binding molecule is detected. An alteration in the formation of the complex in the presence of the agent as compared to in the absence of the agent is indicative of the agent altering the binding of the eotaxin polypeptide to the binding molecule.

Any binding molecule can be used. A preferred binding molecule is an eotaxin receptor. Altering the binding includes, e.g., inhibiting or promoting the binding. The efficacy of the agent can be assessed, e.g., by generating dose response curves from data obtained using various concentrations of the agent. Methods for determining formation of a complex are standard and are known to those skilled in the art. (See, e.g., Tenscher et al., Blood 88:3195 (1996); Rothenberg et al., Mol. Med. 2:334 (1996); Ponath et al., J. Exp. Med. 183:2437 (1996); Daugherty et al., J. Exp. Med. 183:2349 (1996)).

The invention also includes the agent so identified as being able to alter the binding of

eotaxin polypeptide to a binding molecule.

The invention also includes a method for evaluating an agent for the ability to bind to eotaxin polypeptide. An agent is provided. Eotaxin polypeptide is provided. The agent is contacted with the eotaxin polypeptide. The ability of the agent to bind to the eotaxin polypeptide is evaluated. Binding can be determined, e.g., by measuring formation of a complex by standard methods known to those skilled in the art. (See, e.g., Tenscher et al., Blood 88:3195 (1996); Rothenberg et al., Mol. Med. 2:334 (1996); Ponath et al., J. Exp. Med. 183:2437 (1996); Daugherty et al., J. Exp. Med. 183:2349 (1996)).

The invention also includes the agent so identified as being able to bind to eotaxin polypeptide.

The invention also includes a method for evaluating an agent for the ability to bind to a nucleic acid encoding an eotaxin regulatory sequence. An agent is provided. A nucleic acid encoding an eotaxin regulatory sequence is provided. The agent is contacted with the nucleic acid. The ability of the agent to bind to the nucleic acid is evaluated. Binding can be determined, e.g., by measuring formation of a complex by standard methods known to those skilled in the art. Examples of eotaxin regulatory sequences include the N terminus of eotaxin.

The invention also includes the agent so identified as being able to bind to a nucleic acid encoding an eotaxin regulatory sequence.

The following non-limiting examples further illustrate the present invention.

20

EXAMPLES

Example 1: Eotaxin Acts as a Proliferation and Differentiation Factor for Myeloid Progenitors In Vitro

This example illustrates that eotaxin can act in vitro as a proliferation and differentiation factor for myeloid progenitors, inducing the production of granulocytes and macrophages.

Lin⁻ bone marrow cells (Brandt et al., Blood 79:634 (1992); Spangrude et al., Science 241:58 (1988) were purified as follows. Murine bone marrow cells were aspirated from femurs of either BALB/c mice age 3-4 weeks (Jackson Laboratory, Bar Harbor, ME) or GM-CSF deficient mice. Erythrocytes were lysed in ammonium chloride lysis buffer and then the cells were washed twice with PBS. The Lin⁻ cells were selected by the procedures described in Dubois et al., Blood 83:3138-3145 (1994), and Spangrude et al., Science 241:58-62 (1988). Briefly, 0.5 µg/1x10⁶ cells of Rat anti-mouse antibodies specific for RB6-8C5 (GR-1), RA3-6B2

(B220), MAC-1, Lyl-2 (CD-8), L3T4 (CD-4), and Ter-119 (all obtained from Pharmingen, San Diego, CA), were added to bone marrow cells and incubated for 20 minutes at 4°C in PBS supplemented with 2% FBS (PBS/FBS). The cells were then washed and twice centrifuged, and resuspended in 3.5 ml PBS/FBS medium. Sheep anti-rat IgG immunomagnetic beads (DynaI, Oslo, Norway) were then added to the cell suspension at a bead to target-cell ratio of 40:1 and incubated for 20 minutes at 4°C with constant rotation. The cells were magnetically separated with a particle concentrator (DynaI) and Lin⁻ cells were then washed with PBS/FBS and resuspended in IMDM supplemented with 10% FBS.

Thymidine incorporation was used as a short term proliferation assay. Lin⁻ cells were plated into 96-well microtiter plates at a density of 10⁴ cells/well in IMDM media supplemented with 10% FBS and 5x10⁻⁶ mol/L 2-β-mercaptoethanol, 1 μCi [³H]-thymidine (Amersham, UK), and different growth factors. Cultures were incubated for 24 hrs at 37°C in 5% CO₂, and the cells were then harvested with a multiple-sample harvester (Tomtec, Inc., Hamden, CT) and their radioactivity was assessed by a liquid scintillation counter (Wallac Betaplate™, Wallac Oy, Helsinki, Finland; Gaithersburg, MD). Thymidine incorporation was measured as counts per minute/well. See Verfaillie et al., J. Exp. Med. 179:643-649 (1994). Lin⁻ cells proliferating in culture were seeded in 24 well plates and incubated at 37°C in 5% CO₂ for 6 days. It was found that eotaxin stimulated the short term proliferation of Lin⁻ cells. Stem cell factor (SCF), GM-CSF and IL-3 also stimulated the Lin⁻ cells. When eotaxin was added to cells stimulated with SCF, it induced an additive proliferation effect, but it had no additive effect on the proliferation of cells stimulated by GM-CSF or IL-3. MIP-1α, transforming growth factor β (TGF-β), and tumor necrosis factor α (TNF-α), had no proliferation effect on the Lin⁻ cells. When any of the growth factors MIP-1α, TGF-β or TNF-α was added to cells stimulated by the combination of SCF and IL-3, they inhibited their proliferation (Keller et al., Blood 84:2175-2181 (1994); Mayani et al., Exp. Hematol. 23:422-427 (1995)). Recombinant mouse stem cell factor (rmSCF) (Genzyme, Cambridge, MA), rm IL-3 (Pepro Tech, Rocky Hill, NJ), rmGM-CSF (Immunex, Seattle, WA), rmMIP-1α (R&D, Minneapolis, MN), rmEotaxin, lot 095683, lot I155(D) and lot I155(M), (Pepro Tech, Rocky Hill, NJ) were all used in the concentration of 100 ng/ml. rmIL-5 was used in the concentration of 20 ng/ml (R&D), rhTGF-β (British Bio-technology, Oxon) and rmTNF-α (Genentech, San Francisco, CA) were used in the concentrations of 10 ng/ml and 50 ng/ml, respectively.

The long term effect(s) of eotaxin on the proliferation and differentiation of bone marrow

hematopoietic progenitors was further analyzed by growing the cells in the presence of eotaxin. 5×10^3 Lin⁻ cells stimulated with SCF, eotaxin, or the combination of SCF and eotaxin, were seeded in 24 well plates. The number of cells per well, as well as the expression of the granulocyte and macrophage cell surface differentiation markers GR-1 and MAC-1 was tested daily by harvesting the proliferating cells in the tissue culture plates and staining them with antibodies to these antigens. Eotaxin induced the proliferation of Lin⁻ hematopoietic progenitors for up to four days. This proliferation response was coupled with terminal differentiation of the Lin⁻ cells into macrophages which express the differentiation cell surface marker MAC-1 (34%), and granulocytes which express the differentiation cell surface markers MAC-1 and GR-1 (32%). In contrast, most of the cells proliferating in response to SCF, used here as a control, maintained their blast morphology, and did not express either MAC-1 or GR-1 (79%). In agreement with the short term proliferation assay described above, addition of eotaxin to cells stimulated with SCF induced an additive effect on the expansion and differentiation of the MAC-1⁺ (25%) and MAC-1⁺/GR-1⁺ (12%) hematopoietic cells that were found in the cultures after 4-6 days.

Eotaxin was also tested for its ability to stimulate the formation of GM-colonies in methylcellulose. Purified Lin⁻ cells were used to perform a granulocyte-macrophage colony forming unit (GM-CFU) assay as follows. Lin⁻ cells (5×10^3 /plate) or bone marrow cells (10^5 /plate) were cultured in methylcellulose (0.9%) containing Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, MD) supplemented with 20% fetal bovine serum (FBS; Intergen, Purchase, NY) and different growth factors, and incubated at 37°C in 5% CO₂ for 7 days. The growth factors were used in the concentrations described above. Cells from all the colonies in each plate were collected at day 7 and then washed twice with PBS. The cells were then counted (cells/plate), cytopspined and stained with Giemsa, or analyzed for cell surface markers using flow cytometry. The number of cells per colony was calculated by dividing the number of cells per plate by the number of colonies per plate. It was found that eotaxin acts as a granulocyte-macrophage colony stimulating factor for Lin⁻ cells, in the range between about 25 to about 100 ng/ml. Eotaxin has been shown to induce chemotaxis of eosinophils *in vitro* at the same concentration range (Rothenberg et al., Molecular Medicine 2:334-348 (1996)). To rule out an indirect colony stimulating effect by eotaxin through the activation of the Lin⁻ progenitors and secretion of colony stimulating factors by more mature cells, as few as 100 cells per plate were seeded. 2-3 colonies were counted in such plates, indicating that the colony stimulating

activity of eotaxin (at 100 ng/ml) was direct. Eotaxin stimulated the same number of colonies as GM-CSF, but eotaxin colonies were smaller and the number of cells per colony was between 2 to 5 fold lower (eotaxin/GM-CSF, ~500-1000/~2000-3000 cells per colony). In control colony assays, both IL-5 and SCF stimulated few colonies with a low number of cells per colony.

5 However, when SCF or IL-5 were added together with eotaxin in the colony assay, SCF, but not IL-5, had an additive effect on the number of GM colonies stimulated by eotaxin. The effect of SCF on the number of cells per colony and the size of the colonies induced by eotaxin was synergistic. In agreement with the short term proliferation assay, eotaxin did not significantly modify the number of colonies or the number of cells per colony induced by GM-CSF or IL-3.

10 To rule out a possible autocrine GM-CSF secretion by cells stimulated with eotaxin, Lin⁻ progenitors purified from the bone marrow of GM-CSF deficient mice (KO)(3) were studied. There was no difference in the GM colony stimulating activity of eotaxin on either Lin⁻ progenitors purified from wild-type or GM-CSF deficient mice. There also was no difference in the GM colony stimulating activity of eotaxin when neutralizing antibodies to IL-3 were used in
15 these assays. In addition, eotaxin was either treated with (i) 1mM DTT or acetonitrile, (ii) boiled for 5 min, or (iii) mixed with 1:100 polyclonal antibodies to eotaxin. As a control, antibodies against eotaxin were also mixed with GM-CSF. The treated and untreated eotaxin and GM-CSF were then used to perform a GM-CFU assay using Lin⁻ cells that were purified from bone marrow of wild type BALB/c mice. The colony stimulating activity of eotaxin was inhibited by
20 treatments: (i), (ii), or (iii). Furthermore, it was found that Lin⁻ cells expressed the eotaxin receptor, CCR-3 (Ponath et al., J. of Exp. Medicine 183:2437-2448 (1996); Daugherty et al., J. of Exp. Medicine 183:2349-2354 (1996)), and that the stimulatory activity of eotaxin could be blocked ($75 \pm 8.3\%$) by Pertusis toxin.

Further indication for the specificity of eotaxin was demonstrated when the type of cells
25 produced in the colonies stimulated by eotaxin was analyzed and compared to those stimulated by GM-CSF. Cells that were collected from colonies stimulated with eotaxin or GM-CSF were washed with PBS and then stained either with Giemsa or analyzed by flow cytometry. To determine the type of cells in the GM-colonies or tissue culture plates, cells were applied to glass slides by cytocentrifugation (10^5 cells/slide), air dried for 10 minutes and then immersed in
30 Giemsa stain (Sigma, St. Louis, MO), rinsed with distilled water, air dried, and mounted. For immunofluorescence staining, cells were stained with FITC/PE labeled monoclonal antibodies specific for MAC-1 and GR-1 (PharMingen, San Diego, CA). Briefly, 10^5 cells were washed

with staining buffer (0.1% BSA, PBS, 0.02% sodium azide) and incubated with 10 µg/ml (1:50) of purified anti-mouse CD16/CD32(FcR), (PharMingen) for 20 minutes at 4°C. Cells were then washed with staining buffer and the labeled antibodies were added at a dilution of 1:50 for 20 minutes at 4°C. The stained cells were washed twice and analyzed by FACScan flow cytometer using Cell Quest software (Becton Dickinson, CA). The forward scatter and the side scatter of the cells produced in these colonies was determined. Both eotaxin and GM-CSF stimulated the production of highly vacuolated macrophages that express the cell surface integrin MAC-1. However, whereas eotaxin stimulated the production of neutrophilic-metamyelocytes that express the cell surface markers MAC-1 and GR-1, GM-CSF stimulated mostly the production of segmented neutrophils that had higher forward scatter and which express GR-1 and MAC-1.

Example 2: Eotaxin Acts as a Proliferation and Differentiation Factor for Myeloid Progenitors In Vivo

This example illustrates that eotaxin can act in vivo as a proliferation and differentiation factor for myeloid progenitors.

The proliferation and differentiation of bone marrow hematopoietic progenitors was studied using a mouse model of lung allergic inflammation (Gonzalo et al., J. Clin. Invest. 98:1-14 (1996); Gonzalo et al., Immunity 4:1-14 (1996)). Eight to ten week old male and female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Pulmonary eosinophilia in response to ovalbumin (OVA; Sigma, St. Louis, MO) was generated in these mice as described in Gonzalo et al., J. Clin. Invest. 98:1-14 (1996) and Gonzalo et al., Immunity 4:1-14 (1996). Briefly, mice were sensitized with intraperitoneal OVA (0.1 mg/mouse) on day 1 followed by exposure to aerosolized antigen (2% OVA for 5 min on day 8 and 1% OVA for 20 min on days 15-21) to induce the response. At different times after allergen challenge, animals were euthanized by barbiturate overdose and analyzed. Phosphate buffered saline (PBS) was administered (ip. and aerosolized) to mice as a negative control. During the inflammatory phase there was an increase in the number of granulocyte-macrophage colony forming units (GM-CFU) in the bone marrow. During this period, there also was an increase in the percentage of neutrophils in the blood from 12% at day 1 of the treatment to 36% by day 21. During this inflammatory phase, eotaxin expression in the lung increased concomitant with an increase in its serum concentration.

Blocking experiments were performed by injecting the mice with neutralizing polyclonal

antibody against murine eotaxin (20 μ g/mouse, i.v.) (Gonzalo et al., J. Clin. Invest. 98:1-14 (1996)) 30 minutes before OVA administration from day 15 to day 21 and then analyzed 3 hours after allergen challenge. OVA-treated control mice were injected with the same amount of control Ab (Rabbit Immunoglobulin fraction, DAKO, CA) at the same time points indicated during treatment. No endotoxin contamination was detected in any of the reagents used, as assessed by LAL assay (BioWhittaker, MD). The intravenous administration of the neutralizing antibodies to eotaxin during the inflammatory phase totally blocked the increase in the number of granulocyte-macrophage colony forming units (GM-CFU) in the bone marrow. This result indicates a role for eotaxin in stimulating the production of myeloid cells in the bone marrow during lung inflammation.

Further analysis of the in vivo effects of eotaxin was accomplished by i.v. injection of recombinant eotaxin into BALB/c mice. Recombinant mEotaxin (3 μ /mouse) was injected i.v. into BALB/c mice; 48 hr. later the bone marrow cells were collected and a standard GM-CFU assay was performed as described above. Two days after the administration of eotaxin, an increase in the number of GM-CFU in the bone marrow was detected.

In sum, during inflammatory processes, eotaxin stimulates the production of myeloid progenitors in bone marrow.

Example 3: Eotaxin Accelerates the Differentiation of Mast Cell Progenitors

This example illustrates that eotaxin induces colony formation and differentiation of mast cell progenitors in fetal blood.

The ability of eotaxin to induce or inhibit colony formation and/or the differentiation of yolk sac (YS), fetal blood (FBL) and fetal liver (FL) progenitors in methylcellulose cultures was tested in vitro. Cells were grown with SCF (20 ng/ml), eotaxin (50 ng/ml), or eotaxin combined with SCF. The total number of erythroid (BFU-E) and myeloid colonies (CFU-GM, -MIX) was tabulated and cell morphology was assessed by cytopspin preparation. (Aiuti et al., J. Exp. Med. 185:111-120 (1997)). More terminally differentiated mast cells were present in methylcellulose cultures grown only in the presence of both factors. Their identity was confirmed by several different experiments.

They contained prominent toluidine blue (TB)-positive granules, a specific marker of mature mast cells and basophils (Kirshenbaum et al., J. Immunol. 148:772-777 (1992), and chloroacetate esterase, an enzyme found in cells of granulocytic lineage, including mast cells, but

not eosinophils nor basophils. (Yam et al., Am. J. Clin. Pathol. 55:283-290 (1971)). The number of granules per cell was variable, as was cell size, nuclear profile (both mononuclear and multinuclear granulated cells were seen) and the nuclear:cytoplasmic ratio. In three different experiments, the percentage of granulated cells in 11dpc FBL colonies was 10.6%, 5.2% and 8.3% ($x = 8.0 \pm 2.7$); YS cultures contained $2.8\% \pm 1.7$ TB⁺ cells. In cultures of 10dpc YS, the number of TB⁺ cells is much greater: 15% and 28% in two experiments.

In addition, they also expressed c-kit, the receptor for SCF. (Besmer et al., Development 193 Sup:125-137 (1993)). Cytospins of 11dpc YS- and FBL-derived colonies grown in the presence of either factor alone, or both factors combined, were immunostained with the anti-c-kit MAb, ACK2. (Nishikawa et al., EMBO J. 10:2111-2118 (1991)). ACK2⁺ cells were identified (6% for both tissues) only in the populations induced by SCF plus eotaxin.

The presence of mast cell specific serine proteases by RT-PCR (Gonzalo et al., Immunity 4:1-14 (1996)) was assessed. Mast cell granules contain eight different serine proteases and an exopeptidase, carboxypeptidase A. Stevens et al., Proc. Natl. Acad. Sci. USA 91:128-132 (1994). Their expression pattern can be used to characterize mast cells isolated from different tissues or strains under normal or pathological conditions. Immature mast cells grown in vitro can express variable combinations of proteases, depending upon the cytokines they are exposed to and the mouse strain. Thus, cytokines may qualitatively (and quantitatively) affect mast cell differentiation. Bone marrow-derived mast cells (BMMC) cultured in liquid medium for 4-6 weeks, either with SCF or with 20% spleen conditioned medium (CM), expressed mMCP-2 and mMC-CPA, but mMCP-4 was expressed only by mast cells cultured with SCF plus eotaxin.

By RT-PCR, three mast cell proteases in colonies induced by eotaxin alone were not detected. mMC-CPA and mMCP-2 were induced by SCF alone, although mature mast cells were not identified on cytopins prepared from the same populations that the RNA was isolated from. Thus, less differentiated mast cells, containing TB negative or weakly reactive granules may be induced by SCF alone in this culture system, but eotaxin is required for differentiation to the more mature, granulated cells seen on cytopins. In contrast, mMCP-4, which is not inducible by IL-3 in suspension cultures, could only be detected if both factors were present, indicating that eotaxin can affect the phenotype of mast cells in the presence of SCF. The expression of these three serine proteases can be seen in serosal mast cells in WBB6F₁/J- mice, however strain specific differences in the expression of mMCP-2 exist.

Most differentiated mast cells were absent on cytopins prepared from colonies grown in

the presence of both factors plus pertussis toxin (100 ng/ml) and the toxin partially blocked the expression of the mast cell proteases, particularly mMCP-4, supporting the conclusion that eotaxin's synergistic effect on mast differentiation is mediated through a CCR-3 induced signal pathway. Pertussis toxin is an inhibitor of chemokine-induced signaling and chemotaxis

5 (Bargatze et al., J. Exp. Med. 178:367-372 (1993)).

Mast cells were most prevalent in FBL cultures ($8.0\% \pm 2.7\%$), compared to FL at 11 dcp ($0.38\% \pm 0.3$). This was particularly striking, given that FL contains ten times more multipotent precursors (for granulocytes, macrophages and megakaryocytes) than FBL, at embryonic day 11. (Delassus and Cumano, Immunity 4:97-106 (1996)). Four times more colonies were produced
10 from FL in the presence of SCF plus eotaxin than from FBL or YS.

Example 4: Eotaxin Induces Colony Formation Synergistically with SCF

This example illustrates that eotaxin acts synergistically with SCF to accelerate the differentiation of mast cell progenitors.

15 With eotaxin and SCF present, a synergistic growth response was obtained with FL, while an additive effect was seen in YS and FBL. The synergistic effect was not always represented by the colony count, but was seen when the total number of cells produced in methylcellulose cultures was counted. In three experiments, cell counts were obtained that were 1.61, 1.67 and 1.68 times greater than the sum of [cells grown in the presence of eotaxin alone] +
20 [cells grown in the presence of SCF alone]. Pertussis toxin decreased the cytospun cell count by 52% (544 cells/25 fields versus 265 cells with pertussis toxin) and the colony count by 30% ($\pm 8.7\%$) for FBL cultures, in three experiments.

The surface phenotype of the cells produced was analyzed by direct immunofluorescence staining with lineage-specific monoclonal antibodies (MAb) to myeloid cells (Mac-1; CD11b
25 (Springer et al., Eur. J. Immunol. 9:301-306 (1979)), red blood cells (TER-119) (Ikuta et al., Cell 62:83-874 (1990)), and granulocytes (Gr-1) (Fleming et al., J. Immunol. 151:2399-2408 (1993)). In four out of six experiments, there was a synergistic increase of 1.63 times (± 0.4) in the number of Mac-1⁺ cells from colonies grown in the presence of both factors. Mononuclear cells of variable size were the predominant cells induced with SCF and/or eotaxin. The combined
30 percentage of lineage-positive cells was always less than 100%, ranging from 15% to 51%. Thus, some of the lineage-negative cells may be progenitors, mast cells or stromal cells. As discussed above, in the adult mouse, eotaxin in combination with stem cell factor functions as a

granulocyte-macrophage-colony stimulating factor. The increase in Mac-1⁺ cells in the cultures described in the instant Example indicates that eotaxin also promotes myelopoiesis from embryonic progenitors.

5 Example 5: Treating an Individual Having an Allergic Inflammation with Antibodies for Eotaxin

This example illustrates a method for treating a patient having an allergic inflammation with an eotaxin monoclonal antibody so as to reduce the levels of myeloid cells in the patient.

- 10 Eotaxin monoclonal antibody (Gonzalo et al., J. Clin. Invest. 98:1 (1996) is administered to the patient intravenously as a bolus or as an injection over 12 hours, at a concentration of 10 mg/kg body weight. Such administrations are repeated monthly for six months, resulting in treatment of the allergic inflammation.

15 Example 6: Treating an Individual Having Myeloid Leukemia with Eotaxin

This example illustrates a method for treating a patient having myeloid leukemia with eotaxin so as to increase the levels of myeloid cells in the patient. Eotaxin is obtained (Pepro Tech, Rocky Hill, NJ). The eotaxin is administered to the patient by injection intravenously, once a day, at a concentration of 5 mg/kg body weight. Such administrations are repeated

- 20 weekly for three months, resulting in treatment of the myeloid leukemia.

Those skilled in the art will be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for determining if a mammal is at risk for a disease associated with abnormal levels of myeloid cells, comprising:

providing a mammal; and

5 evaluating an aspect of eotaxin metabolism or structure in said mammal, an abnormality in said aspect of eotaxin metabolism or structure being diagnostic of being at risk for a disease associated with abnormal levels of myeloid cells.

2. The method of claim 1 wherein said disease is an autoimmune or inflammatory
10 disease.

3. The method of claim 2 wherein said disease is selected from the group consisting of allergic inflammation, systemic lupus erythematosus and rheumatoid arthritis.

15 4. The method of claim 1 wherein said disease is associated with decreased levels of myeloid cells.

5. The method of claim 4 wherein said disease is selected from the group consisting of myeloid leukemia, conditions from myeloablative treatments, mast cell deficiencies and
20 myelodysplastic syndromes.

6. The method of claim 1 wherein said myeloid cells are mast cells.

7. The method of claim 1 wherein said myeloid cells are selected from the group
25 consisting of granulocytes, macrophages, and combinations thereof.

8. The method of claim 1 wherein said abnormality in said aspect of metabolism or structure is a mutation in a gene encoding said eotaxin or a receptor of said eotaxin.

30 9. The method of claim 1 wherein said abnormality in said aspect of metabolism or structure is abnormal polypeptide or RNA levels of said eotaxin or of a receptor of said eotaxin.

10. A method for evaluating an agent for use in modulating the level of myeloid cells in a mammal, comprising:

providing a mammalian cell or mammal;

providing an agent;

5 administering said agent to said cell or mammal in a therapeutically effective amount;

and

evaluating the effect of said agent on an aspect of eotaxin metabolism, a change in said aspect being indicative of the usefulness of said agent in modulating the level of myeloid cells in a mammal.

10

11. The method of claim 10 wherein said mammalian cell or mammal that is provided has a wild type pattern of eotaxin metabolism.

12. The method of claim 10 wherein said mammalian cell or mammal that is provided
15 has a non-wild type pattern of eotaxin metabolism.

13. The method of claim 12 wherein said non-wild type pattern results from overexpression or underexpression of said eotaxin.

20 14. The method of claim 10 wherein said aspect of eotaxin metabolism is a parameter related to the level of myeloid cells.

15. The method of claim 10 wherein said agent is administered to said mammalian cell in vitro, and if said change in said aspect of eotaxin metabolism occurs, then further
25 administering said agent to a mammal in a therapeutically effective amount and evaluating the in vivo effect of said agent on an aspect of eotaxin metabolism, a change in said aspect being indicative of the usefulness of said agent in modulating the level of myeloid cells in a mammal.

30 16. The method of claim 10 wherein said cell is a transgenic cell and said mammal is a non-human transgenic mammal.

17. The method of claim 10 wherein said myeloid cells are mast cells.

18. The method of claim 10 wherein said myeloid cells are selected from the group consisting of granulocytes, macrophages, and combinations thereof.

19. The method of claim 10 further comprising providing stem cell factor or a
5 biologically active fragment or analog thereof and administering said stem cell factor or a biologically active fragment or analog thereof to said cell or mammal in a therapeutically effective amount.

20. The agent identified in claim 10.

21. A method for treating a disease associated with high levels of myeloid cells in a mammal, comprising:

providing a mammal in need of treatment for a disease associated with high levels of myeloid cells;

15 providing an agent capable of altering an aspect of eotaxin metabolism or structure; and administering said agent to said mammal in a therapeutically effective amount such that treatment of said disease associated with high levels of myeloid cells in said mammal occurs.

22. The method of claim 21 wherein said disease is an autoimmune or inflammatory
20 disease.

23. The method of claim 22 wherein said disease is selected from the group consisting of allergic inflammation, systemic lupus erythematosus and rheumatoid arthritis.

24. The method of claim 21 wherein said myeloid cells are mast cells.

25. The method of claim 21 wherein said myeloid cells are selected from the group consisting of granulocytes, macrophages, and combinations thereof.

26. The method of claim 21 wherein said agent binds to eotaxin.

27. The method of claim 21 wherein said agent is a natural ligand for eotaxin.

28. The method of claim 21 wherein said agent is an artificial ligand for eotaxin.

29. The method of claim 21 wherein said agent is an antagonist or an agonist.

5 30. The method of claim 21 wherein said agent inhibits the interaction of eotaxin with a binding molecule.

31. The method of claim 21 wherein said agent is an inhibitor of a molecule that induces the expression of eotaxin.

10

32. The method of claim 21 wherein said agent is an antibody.

33. The method of claim 21 wherein said agent is selected from the group consisting of a mimetic of eotaxin and a mimetic of a binding molecule of eotaxin.

15

34. The method of claim 21 wherein said agent is an antisense nucleic acid.

35. The method of claim 21 wherein said agent comprises a nucleic acid encoding an eotaxin regulatory sequence or a biologically active fragment thereof.

20

36. The method of claim 21 wherein said agent further is capable of altering an aspect of stem cell factor metabolism or structure.

37. A method for treating a disease associated with low levels of myeloid cells,

25 comprising:

providing a mammal in need of treatment for a disease associated with low levels of myeloid cells;

providing eotaxin or a biologically active analog or fragment thereof; and

administering said eotaxin or biologically active analog or fragment thereof to said

30

mammal in a therapeutically effective amount such that treatment of said disease associated with low levels of myeloid cells occurs.

38. The method of claim 37 wherein said disease is selected from the group consisting of myeloid leukemia, conditions from myeloablative treatments, mast cell deficiencies and myelodysplastic syndromes.

5 39. The method of claim 37 wherein said myeloid cells are mast cells.

40. The method of claim 37 wherein said myeloid cells are selected from the group consisting of granulocytes, macrophages, and combinations thereof.

10 41. The method of claim 37 further comprising providing stem cell factor or biologically active fragment or analog thereof, and administering said stem cell factor or biologically active fragment or analog thereof to said mammal in a therapeutically effective amount.

15 42. A method for altering the proliferation or differentiation of myeloid progenitors in a mammal, comprising:

providing a mammal having myeloid progenitors, said mammal being in need of altering the proliferation or differentiation of said myeloid progenitors;

providing a compound selected from the group consisting of eotaxin, a biologically active fragment thereof, a biologically active analog thereof, an antagonist and an agonist; and

20 administering said compound to said mammal under conditions which allow said compound to alter the proliferation or differentiation of said myeloid progenitors.

43. The method of claim 42 wherein the altering of the proliferation or differentiation of said myeloid progenitors comprises increased production of macrophages or granulocytes.

25 44. The method of claim 42 further comprising providing stem cell factor or a biologically active fragment or analog thereof, and administering said stem cell factor or biologically active fragment or analog thereof to said mammal.

30 45. The method of claim 42 wherein said myeloid progenitors are mast cell progenitors.

46. A method for monitoring a therapeutic treatment for a disease affecting the level of myeloid cells in a mammal, said disease being associated with abnormal levels of an aspect of eotaxin metabolism in said mammal, comprising:

5 evaluating the levels of an aspect of eotaxin metabolism in a plurality of biological samples obtained at different time points from a mammal undergoing a therapeutic treatment for a disease affecting the level of myeloid cells, said disease being associated with abnormal levels of an aspect of eotaxin metabolism in said mammal.

47. The method of claim 46 wherein said myeloid cells are mast cells.

10

48. The method of claim 46 wherein said myeloid cells are selected from the group consisting of granulocytes, macrophages, and combinations thereof.

49. A pharmaceutical composition for treating a disease associated with low levels of myeloid cells in a mammal, comprising:

15 a therapeutically effective amount of eotaxin or a biologically active fragment or analog thereof, said eotaxin or biologically active fragment or analog thereof being capable of stimulating the proliferation or differentiation of myeloid progenitors so as to result in treatment of said disease associated with low levels of myeloid cells in said mammal; and

20 a pharmaceutically acceptable carrier.

50. The pharmaceutical composition of claim 49 further comprising stem cell factor or a biologically active fragment or analog thereof, said stem cell factor or biologically active fragment or analog thereof being capable of increasing the stimulation of the proliferation or differentiation of said myeloid progenitors by said eotaxin or biologically active fragment or analog thereof.

25

51. The pharmaceutical composition of claim 49 wherein said myeloid cells are mast cells.

30

52. A pharmaceutical composition for treating an autoimmune or inflammatory disease in a mammal, comprising:

a therapeutically effective amount of an agent, said agent being capable of altering an aspect of eotaxin metabolism or structure in said mammal so as to result in treatment of said autoimmune or inflammatory disease in said mammal; and
a pharmaceutically acceptable carrier.

5

53. The pharmaceutical composition of claim 52 wherein said agent is an antagonist of eotaxin.

54. The pharmaceutical composition of claim 53 wherein said antagonist is selected from
10 the group consisting of an antibody for eotaxin, a fragment of eotaxin, an analog of eotaxin, a small-molecule antagonist of eotaxin, a mimetic of eotaxin, an antisense molecule for eotaxin and a binding molecule for eotaxin.

55. A method of making an altered eotaxin polypeptide having an antagonist or agonist
15 activity so as to alter the proliferation or differentiation of myeloid progenitors in a mammal, comprising:

providing an eotaxin polypeptide;

altering the amino acid sequence of said eotaxin polypeptide; and

testing said altered polypeptide for an effect on the proliferation or differentiation of
20 myeloid progenitors, a change in said proliferation or differentiation being indicative of an eotaxin polypeptide having an antagonist or agonist activity.

56. The method of claim 55 wherein said myeloid progenitors are mast cell progenitors.

25 57. A method for evaluating an agent for the ability to alter the binding of eotaxin polypeptide to a binding molecule, comprising:

providing an agent;

providing eotaxin polypeptide;

providing a binding molecule;

30 combining said agent, said eotaxin polypeptide and said binding molecule; and

detecting formation of a complex comprising said eotaxin polypeptide and said binding molecule, an alteration in formation of said complex in the presence of said agent as compared to

in the absence of said agent being indicative of said agent altering the binding of said eotaxin polypeptide to said binding molecule.

58. A method for evaluating an agent for the ability to bind to eotaxin polypeptide,
5 comprising:
 providing an agent;
 providing eotaxin polypeptide;
 contacting said agent with said eotaxin polypeptide; and
 evaluating the ability of said agent to bind to said eotaxin polypeptide.

10

59. A method for evaluating an agent for the ability to bind to a nucleic acid and
encoding an eotaxin regulatory sequence, comprising:

- providing an agent;
 providing a nucleic acid encoding an eotaxin regulatory sequence;
15 contacting said agent with said nucleic acid; and
 evaluating the ability of said agent to bind to said nucleic acid.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17280

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; G01N 33/53

US CL :435/6, 7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS

search terms: eotaxin, eosinophil#, chemokine#, chemotactic, chemoattractant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 97/00960 A1 (LEUKOSITE, INC.) 09 January 1997, page 5, lines 11-15, page 27, lines 14-20, page 27, line 33 to page 28, line 4, page 48, line 22 to page 49, line 25.	1-3, 6, 7 --- 46, 47
X	KAMIL et al. Eotaxin mRNA expression in chronic sinusitis and allergen-induced late nasal responses. Journal of Allergy and Clinical Immunology. January 1997, Vol. 99, No. 1, Part 2, page S421, abstract 1711, see entire abstract.	1-3, 6, 7, 9
X	RENZI et al. Eotaxin is increased in the airways and bronchoalveolar lavage of asthmatic patients. Journal of Allergy and Clinical Immunology. January 1997, Vol. 99, No. 1, Part 2, page S364, abstract 1486, see entire document.	1-3, 6, 7, 9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

09 NOVEMBER 1998

Date of mailing of the international search report

22 JAN 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SCOTT D. PRIEBE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US98/17280

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAMKHIOUED et al. Eotaxin is present in the BAL fluid of asthmatics and induces normal eosinophil migration: Effects of priming with IL-5. Journal of Allergy and Clinical Immunology. January 1997, Vol. 99, No. 1, Part 2, page S170, abstract 687, see entire document.	1-3, 6, 7
X	MATTOLI et al. Eotaxin expression and eosinophilic inflammation in asthma. Biochemical and Biophysical Research Communications. 18 July 1997, Vol. 236, pages 299-301, see entire document.	1-3, 6, 7, 9
X	GARCIA-ZEPEDA et al. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. Nature Medicine. April 1996, Vol. 2, No. 4, pages 449-456, see entire document.	1-3, 6, 7, 9
A	PONATH et al. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. Journal of Experimental Medicine. 01 June 1996, Vol. 183, No. 6, pages 2437-2448.	1-9, 46-48
A	KITAURA et al. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. Journal of Biological Chemistry. 29 March 1996, Vol. 271, No. 13, pages 7725-7730.	1-9, 46-48
A	GONZALO et al. Mouse eotaxin expression parallels eosinophil accumulation during lung allergic inflammation but it is not restricted to a Th2-type response. Immunity. January 1996, Vol. 4, pages 1-14.	1-9, 46-48

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/17280

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9,46-48

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17280

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-9, 46-48, drawn to a method for monitoring eotaxin metabolism in a mammal.

Group II, claim(s) 10-19, 55, 56, drawn to method for identification of an agent which modulates eotaxin metabolism using cells or mammals.

Group III, claim(s) 20, drawn to unspecified agent that modifies eotaxin metabolism.

Group IV, claim(s) 21-36, 42, 52-54, drawn to method for decreasing level of myeloid cells and pharmaceutical composition therefore.

Group V, claim(s) 37-45, 49-51, drawn to drawn to method for increasing level of myeloid cells and pharmaceutical composition therefore.

Group VI, claim(s) 57, drawn to method for identification of agent which alters binding of eotaxin and unspecified eotaxin ligand.

Group VII, claim(s) 58, drawn to method for identification of ligand that binds eotaxin.

Group VIII, claim(s) 59, drawn to method for identification of agent that binds eotaxin regulatory sequence in nucleic acid.

As written, claim 42 embraces two distinct methods, method for increasing eotaxin levels and method for decreasing eotaxin levels. Each aspect of claim 42 will be separately examined should the additional search fees be paid for examination of either of groups IV or V

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species relate to group IV and are as follows: antibody for eotaxin, fragment of eotaxin, analog of eotaxin, small molecule antagonist of eotaxin, mimetic of eotaxin, antisense nucleic acid for eotaxin gene, sequestering agent for eotaxin.

The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Each of the methods relates to various, distinct aspects of eotaxin. As disclosed in the description, eotaxin was previously known in the prior art and the coding sequence for human eotaxin had been cloned by Ponath et al. (J. Clin. Invest. 97: 604-612, 1996). Each of the methods has a different and distinct goal or outcome, and comprises different method steps. Criteria for lack of unity of invention as set forth in 37 USC 1.475(b) do not provide for multiple, distinct methods relating to various related aspects of a product or related products.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each of the agents recited is a physically and biochemically different compound which inhibits the effects of eotaxin by different mechanisms, i.e. inactivating or sequestering eotaxin, inhibiting or sequestering eotaxin receptor or ligand, inhibiting eotaxin expression. There is no relationship between these compounds other than that they directly or indirectly relate to reducing the effects of excess eotaxin levels.